Quantification of Methadone and its Metabolite 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine in Third Instar Larvae of *Lucilia sericata* (Diptera: Calliphoridae) Using Liquid Chromatography–Tandem Mass Spectrometry

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Abstract

Entomotoxicology studies the application of toxicological analysis on necrophageous insects present on human remains. This paper describes the development and validation of a sensitive liquid chromatography–tandem mass spectrometry method for quantification of methadone and its main metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), in developmental stages of *Lucilia sericata*. One single larva was pulverized in a disposable vial and then extracted with 1-chlorobutane. After evaporation of the organic layer, samples were reconstituted in the mobile phase. Chromatographic separation was achieved on a NUCLEODUR® Sphinx RP column with a liquid chromatographic gradient (0.1% formic acid and methanol), ensuring the elution of methadone and EDDP within 15 min. The method was fully validated according to international guidelines. The use of liquid–liquid extraction was demonstrated to be effective (matrix effect < 27% and recovery > 66%). The method was linear over the dynamic range (10–400 pg/mg larva) with excellent within- and between-run precision and bias (CV% < 5%). The lower limit of quantification was fixed at 10 pg/mg larva. No instability of the extracted samples was observed in the autosampler after three freeze/thaw cycles and after two months at –20°C. The validated method was applied to third instar larvae of *Lucilia sericata* reared on beef heart spiked with 4 µg/g methadone and on a postmortem methadone overdose case. The validation and actual sample analysis showed that the method is sensitive, rugged, precise, accurate, and well-suited for routine analysis of methadone and EDDP in a single larva obtained from forensic cases.

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Introduction

Entomotoxicology studies the application of toxicological analysis to carrion-feeding insects in order to identify drugs and toxins present in intoxicated tissues (1). In the absence of any suitable tissues or fluids, insects have been proposed as reliable alternate specimens for forensic purposes. During a comparative study, Kintz et al. (2) obtained greater sensitivity using fly larvae instead of putrefied material. Analysis of larvae can offer technical advantages with respect to human tissue, such as no emulsion during extraction step, less endogenous peaks in chromatograms, and an easy and fast sampling (3). In addition, drug concentrations seem to be stable in insects, although this is not the case for several postmortem tissues (3). Moreover, quantification of drugs in insects is of interest in insect development studies.

Since the first identification in 1980 of phenobarbital in fly larvae found on a skeletonized corpse (4), entomotoxicology has become an established approach (1,3,5), and numerous experiments have been carried out by different groups to detect drugs in larvae, pupae, and puparia. Several techniques have been used for the detection of organic substances in these tissues: immunoassays (7,8), gas chromatography (GC) coupled to a flame-ionization detection (FID) or mass spectrometry (MS) (9–12), liquid chromatography (LC) coupled to diode-array detection (DAD) (13), chemiluminescence (CL) (14), or MS (15–17).

However, given the heterogeneity of insect tissues, the use of non-specific tests (such as immunoassays) is not recommended (3). The possibility of coupling GC or LC with tandem MS increases the sensitivity and selectivity even further, often with much reduced sample preparation and analysis times (18). In-

creased sensitivity can prove highly advantageous for the analysis of arthropod tissues and may enable the measurement of drugs in single larva (18) rather than pools (2,19). The ability to quantify drug levels in individual larvae is particularly useful in investigations concerning the effect of drugs on insect development (18). Moreover, the size of the crop and their contents depend not only on behavioral stages but also on the activity of third instar larvae (20). It is therefore important to analyze a single specimen to avoid large variability in toxicological results due to different pharmacokinetics of species (9) or stages (17). This third instar developmental stage is generally the most represented in a forensic insect sample.

The purpose of this study was to develop and validate an LC–MS–MS method to quantify methadone and its main metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), in third instar larvae of *Lucilia sericata*, a species more common in the temperate areas of the northern hemisphere. This species has a great forensic interest for estimating the postmortem interval. Determination of methadone, a µ-receptor agonist, was chosen because this opioid is often found in fatal cases and it accelerates the development times of *Lucilia sericata* (21). This drug possesses central effects similar to morphine, such as sedation, analgesia, and can result in a fatal respiratory depression. In Belgium, it is licensed as a substitute for opioid addicts, and it is often co-administered with other psychotropic substances such as drugs-of-abuse, ethanol, or benzodiazepines. Finally, this developed method was applied to experimental samples and samples obtained from a forensic case.

Experimental

Reagents

Drug standards of methadone and EDDP (1.0 mg/mL methanol) were purchased from LGC Promochem (Molsheim, France). A mixed stock solution was prepared (4 µg/mL), which was then further diluted with 0.1% aqueous formic acid to yield working solutions at appropriate concentration to add to the calibrator samples.

Deuterated internal standards, methadone-d_9 and EDDP-d_3, were also obtained from LGC Promochem (Molsheim, France) at a concentration of 0.1 mg/mL in methanol. A mixed stock solution was prepared (1 µg/mL) and further diluted with 0.1% aqueous formic acid. A concentration of 50 ng/mL was added to samples and calibrators. Ammonium chloride buffer (powder) and formic acid (98%) were purchased from Sigma Aldrich (St. Louis, MO). Chlorobutane (HPLC grade) was purchased from Merck (Darmstadt, Germany). Water, 0.1% aqueous formic acid/water (ULC–MS grade), and methanol (LC–MS grade) were obtained from Biosolve (Valkenswaard, the Netherlands).

Specimens

Developmental stages of the blowfly *Lucilia sericata* were collected from a single Belgian population around Brussels, Belgium. These flies were maintained in an insectarium at 18–22°C with 60–70% humidity and a photoperiod of Light (L): Dark (D) 16: 8h. Adults were identified using morphological characters (22,23). About 200 flies were kept in gauze cages (35 × 35 × 35 cm) and fed ad libidum with water, sugar, powdered milk, and brewer's yeast. For oviposition, fresh beef heart was provided as the rearing substrate for developing larvae. Mixed beef heart was spiked with 10 mL of distilled water containing methadone concentration of 4 µg/g and mixed in a Waring blender (VWR, Leuven, Belgium) for 5 min at ambient temperature. About 100 newly hatched larvae were deposited and reared on mixed beef heart (250 g) spiked with methadone or on blank beef heart. Each rearing was placed in a plastic box containing sand at 20 ± 1°C (Incubator MIR 553, Sanyo, Osaka, Japan).

At regular time intervals (from 66 to 162 h, every 24 h), specimens were randomly collected from the spiked beef heart. This interval corresponds to third instar larvae.

Third instar larvae were divided by two behavioral distinct stages: feeding (66–90 h) and post-feeding (114–162 h). Once the maximum size is attained, they stop feeding and wander away from the food source in search for a place to become a pupa. Pupae are characterized by a hard and brownish-black coloration cuticle (24).

All specimens were rinsed thoroughly with deionized water and then dried on absorbent filter paper prior to killing. Larvae were killed by freezing (–20ºC) and then stored at this temperature until further analysis.

Sample preparation

A single larva specimen was placed in a 100-µL water-containing disposable vial (Precellys kit MK28 with metal beads, Bertin Technologies, Montigny-Le-Bretonneux, France), using disposable vials for each single larva avoids cross contamination between samples. Thereafter, samples were transformed to powder during 30 s at 4000 rpm in a Precellys 48. The samples were then transferred to a glass vial after addition of 400 µL of water and 500 µL of saturated ammonium chloride buffer (pH 9.2). Thereafter, 4 mL of 1-chlorobutane was added, and mechanical shaking was carried out for 10 min. Following centrifugation at 2333 × *g* for 10 min, the clear organic extract was transferred to a clean vial and evaporated to dryness in a vacuum centrifuge (Jouan, Heverlee, Belgium). Then the samples were reconstituted in 500 µL of aqueous mobile phase.

LC

LC was performed using a Waters Alliance 2690 system (Zellik, Belgium). Chromatography was achieved using an NUCLEODUR Sphinx RP column (3 µm, 50 × 2 mm, FilterService, Eupen, Belgium) eluted with a gradient delivered with 0.1% aqueous formic acid (A) and a methanol/0.1% aqueous formic acid mixture (B) at a flow rate of 250 µL/min. A gradient was applied starting at 20% B for 3 min. Thereafter, B increased gradually to 80% during the next 4 min before returning to the initial conditions. The total run-time was 15 min, and 20 µL of the extract was injected onto the LC system.

MS

A Quattro Ultima triple-quadrupole MS (Waters) fitted with

a Z-Spray ion interface was used for all analyses. Ionization was achieved using electrospray in positive ionization mode (ES+). The following conditions were optimal for the analysis of methadone and EDDP: capillary voltage, 1.0 kV; source block temperature, 120ºC; desolvation gas (nitrogen) heated to 350ºC and delivered at a flow rate of 800 L/h.

In order to establish appropriate multiple reaction monitoring (MRM) conditions for the methadone, EDDP and their respective deuterated analogues [solutions of the standards in 50 ng/mL (0.1% aqueous formic acid/methanol, 50:50, v/v)] were individually infused into the MS and the cone voltage (CV) was adjusted to maximize the intensity of the protonated molecular species $[M+H]^+$. Collision-induced dissociation (CID) of each protonated molecule was performed. Collision gas (argon) pressure was maintained at 2.5×10^{-3} mBar, and the collision energy (eV) was adjusted to optimize the signal for the most abundant product.

All aspects of system operation and data acquisition were controlled using MassLynx V4.1 SCN627 software (Waters).

Method validation

The method was validated based on FDA guidelines (25). Quantification was performed by integration of the area under the specific MRM chromatograms. In all cases methadone and EDDP concentrations were calculated in reference to the integrated area of its respective deuterated analogue, which was added before the extraction procedure. For the recovery experiments, the deuterated analogue is added after extraction. All quantification was performed against a daily prepared calibration curve.

Linearity. Standard curves were generated for methadone and EDDP using least squares linear regression. Assay linearity was investigated by constructing calibration curves (n = 8) ranging from 10 to 400 pg/mg larva (10, 20, 40, 100, 250, 200, and 400 pg/mg larva) for methadone and EDDP.

Intra- and interassay precision and bias. Quality controls (QC) were analyzed at three concentration levels at 28, 160, and 320 pg/mg larva for methadone and EDDP. Within-run and between-run precision were estimated by replicate (n = 2) analysis of the QC samples performed on eight different days by applying one-way analysis of variance (ANOVA) with the varied factor day as the grouping variable (26). The precision was expressed as the variation coefficient (% CV). A comparison of the calculated QC samples concentrations to their respective nominal values was used to assess the accuracy (bias) of the method.

Limit of quantification (LOQ). The LOQ was defined as the concentration of the lowest calibrator that was calculated to be within ± 20% of the nominal value with a %CV less than 20%. The acceptance criteria for ion ratios were equal to or lower than 20%, and retention time deviations were lower than 3.5% relative to that of the corresponding control or calibrator.

Selectivity. The selectivity of the method was verified by examination of the chromatograms obtained after the extraction of nine different blank larvae samples. Moreover, blank larvae samples (n = 3) spiked with 27 benzodiazepines, benzoylecgonine, codeine, cocaine, 6-MAM, morphine, THC, 11-OH-THC, THC-COOH, zolpidem, zopiclone, and zaleplon were also analyzed to check for interferences.

Stability. The stability of the processed sample, when placed in the autosampler (maintained at 6 ± 2°C), was checked by repeated injections of two extracted QC samples at 28 and 320 pg/mg of larva (n = 6). Six extracted samples at each concentration were spiked with the internal standard just before analysis at time zero, 24 h, and after 96 h, and the stabilities were estimated by comparing the peak response ratios at each concentration. The stability was also estimated after three freeze/thaw cycles and after being kept for two months at –18°C (28 and 320 pg/mg, n = 6 each for both experiments).

Extraction recovery experiments/assessment of matrix effects. Extraction recoveries were determined at a concentration 28, 160, and 320 pg/mg larva corresponding respectively at QC low, medium, and high (n = 6) for three developmental stages (larvae, pupae, and adults). Therefore, standard working solutions were spiked in blank matrix samples (extraction samples) before or after sample preparation (control samples).

To assess any potential suppression or enhancement of ionization from components present in the extracted biological matrix, two different analyses were carried out. First, a continuous post-column infusion was performed using a mixture of methadone, EDDP, and deuterated analogues (10 ng/mL at a flow rate of 10 µL/min) to produce a constant elevated response in both MRM channels. The interference of this constant response was monitored following the injection of extracted samples and compared to the response following the injection of mobile phase only. The second experiment consisted of a comparison between the peak responses of the analytes spiked to blank larvae, pupae, and adults at 28, 160, and 320 pg/mg (n = 6) with those obtained after being spiked in the mobile phase at the same concentration levels.

Results and Discussion

Method validation

Selectivity. The applied gradient ensured the elution of all the drugs examined within 15 min and produced chromatographic peaks of acceptable symmetry. Selectivity of the method was achieved by a combination of retention time, precursor, and product ions.

The selectivity of the method was acceptable in terms of absence of interference in the nine analyzed blank samples analyzed. The most prominent precursor-product transitions were used for quantification, and the next most abundant ones were used as qualifiers (Table I). The ion ratios (quantifier/qualifier) were 2.2 and 2.4 for methadone and EDDP, respectively.

For the corresponding deuterated analogues, only one transition was monitored. Injection of single solutions did not produce interference in the other MRM channels. No interferences were observed after the analysis of blank larvae spiked with several over-the-counter drugs, ensuring the selectivity of the method.

Linearity, precision, bias, and LOQ. The linearity of the method was verified over the concentration range 10–400

pg/mg larva. After evaluating different types of regression, the calibration curves were constructed using a 1/*x* weighting for all of the compounds. The study was based on the standard error of the fit and minimization of bias of the calibrators (27). In all cases an $r^2 > 0.99$ was obtained. The lower limit of quantification was fixed at the lowest calibrator in the linearity experiments. Within-run precision (%CV) was lower than 4% for methadone and EDDP at the QC sample concentrations. The between-run precision (%CV) was also < 5%. A bias < 3% was observed for both compounds. The results are summarized in Table II.

Stability, extraction recoveries, and matrix effect. Stability in autosampler, freeze-thaw, and long-term were tested against a lower percentage limit corresponding to 90–110% of the ratio (mean value of stability samples/mean value control samples) with a 90% confidence interval of the stability samples between 80 and 120% of the mean of the control samples. No statistical significant differences and instability could be observed for the two concentrations for methadone and EDDP ($p < 0.05$).

One of the limiting factors of LC–MS–MS applications is the potential presence of matrix effects leading to suppression or enhancement of the analyte response. In third instar larvae, we can conclude that the matrix effects found for methadone and EDDP are still acceptable (< 15%) for both experiments (post-column infusion and extracted versus control samples). For post-column infusion experiments, no significant changes in responses were observed in the chromatographic run. The results confirm the usefulness of the liquid–liquid extraction procedure as a sample clean up before chromatography to obtain reproducible and reliable quantitative results for all compounds without major interference of matrix compounds. The results of the extraction recovery and matrix effect study are presented in Table III.

Experimental samples

The developed method was applied to third instar larvae of *Lucilia sericata* that had been reared on artificial foodstuff (beef heart) spiked with 4 µg/g methadone. Figure 1 represents the results following the analysis of specimens sampled randomly from substrates at regular time intervals. In all cases, both the parent drug and metabolite could be detected following the analysis of a single specimen. Methadone and EDDP were easily quantified in third instar larvae in both feeding and post-feeding stage. A stabilization of concentrations between feeding and post-feeding stage was observed. Although median methadone concentrations between feeding and post-feeding stage seem rather similar, the concentration of its metabolite decreases significantly between both stages. The concentration of EDDP is higher than methadone in feeding stage; however, this seems to reverse in post-feeding stage (Figure 1).

For the moment, the origin of the metabolite cannot be clearly elucidated. Presence of EDDP can be the result of substrate enzymes. However, this metabolite could also result from larvae metabolism (28) and/or from a prolonged elimination of EDDP contained in larvae. Larvae metabolize with

Table I. MRM Transitions and Conditions for the Measurement of Methadone and EDDP and Their Respective Deuterated Analogues

Compound	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Cone Voltage (V)	Collision Energy (eV)
Methadone	310.2	158.9	30	20
	310.2	222.9	30	20
Methadone-d_9	319.4	268.3	50	15
EDDP	278.2	186.0	30	30
	278.2	218.9	30	40
EDDP-d_3	281.2	234.0	30	30

Table II. Within-Run Precision, Between-Run Precision, and Bias*

Compound	Concentration (pg/mg larva)	Within-Run Precision (%CV)	Between-Run Precision (%CV)	Bias (%)
Methadone				
QC Low	28	2.1	4.2	–1.8
QC Med	160	1.8	3.9	2.5
QC High	320	3.2	4.3	0.6
EDDP				
QC Low	28	2.3	4.7	0.2
QC Med	160	2.2	3.4	0.8
QC High	320	1.0	4.0	–0.1

* Expressed as percent of deviation of the QC samples.

Table III. Percentage of Extraction Recovery and Matrix Suppression and Standard Deviation Between Samples*

	Matrix effect (%)			Recovery (%)		
	QC low	QC med	QC high	QC low	QC med	QC high
3rd Larvae						
EDDP	89.0 (4.3)	86.9 (1.9)	95.5 (3.3)	71.3 (12.4)	71.4 (6.7)	67.7 (8.7)
Methadone	83.1 (4.0)	85.3 (1.8)	88.8 (1.2)	79.2 (8.2)	77.1 (4.5)	78.3 (7.5)
Pupae						
EDDP	87.0 (2.6)	86.8 (0.7)	94.7 (3.4)	8.0 (11.6)	84.3 (8.5)	74.6 (12.3)
Methadone	79.0 (2.8)	73.8 (0.8)	85.6 (1.7)	86.1 (7.8)	98.2 (8.3)	77.0 (13.3)
Adults						
EDDP	89.9 (1.2)	86.9 (1.0)	92.2 (1.0)	84.2 (8.3)	88.1 (5.7)	95.3 (7.7)
Methadone	79.0 (1.0)	86.9 (2.0)	84.8 (2.3)	90.1 (14.9)	92.2 (7.4)	96.8 (4.7)

* Standard deviation listed in parentheses.

varying levels of efficiency (29). Unlike methadone, a high decrease of EDDP concentration was observed between feeding and post-feeding third instar stage. This difference on drug elimination could be explained by different chemical structure of compounds. Kharbouche et al. (17) demonstrated that metabolites of opiates have different kinetics in *L. sericata.* This author observed that morphine has a rapid elimination and was not found in puparia, while norcodeine was found in higher concentrations than the parent drug codeine (17). The differences in elimination of these opiates could be related to their chemical structure and pharmacological properties. Elimination rate at the post-feeding stage exceeded the absorption rate inducing a decrease of the opiate concentration in the larvae (10,17). This elimination is not limited to the post-feeding stage but expands over the larval stage (17).

In our study, concentrations of EDDP detected in post-feeding maggots were significantly lower than for feeding maggots. This was not observed for methadone. The metabolite EDDP is more polar and thus more water-soluble and could be excreted more easily by malpighian tubules. Methadone, in contrast, could be accumulated in adipocytes or integuments as already observed for morphine in third instar larvae (30).

Our work confirms studies of Campobasso et al. (10) and Pien et al. (28). As a result, feeding larvae were considered to be the most appropriate sample for the toxicological analysis (17,29). However, a high variability of drug concentrations is still observed. Sadler et al. (29) also show a large degree of biological variation in larval drug concentrations in larvae of *Calliphora vicina.* Similarly, Definis-Gojanović et al. (11) have shown that the accumulation of drugs was unpredictable. In fact, this variability could be due to three physiologic states of larvae (20). Thus, it is possible to distinguish three main behaviors (feeding, looking for food, and digestion) in relation with residues in the crop (20). Drug concentration could vary between these different states. Therefore, the high variability could be due to different kinetics of detoxification or process of feeding/excretion between larval specimens. Although larvae

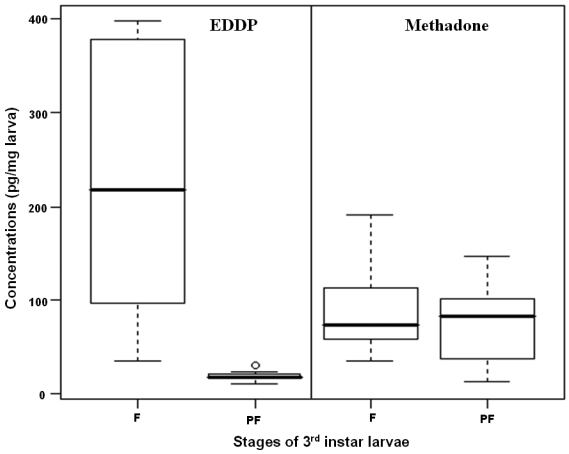

Figure 1. Boxplots of concentrations (pg/mg larva) of EDDP and methadone in 3rd instar larvae during distinct behaviorally stages (F, feeding; PF, post-feeding). Numbers of specimens vary in post-feeding from 9 (EDDP) to 12 (methadone) and 10 in feeding stages for two compounds. Only traces were observed in three specimens for EDDP in post-feeding stages.

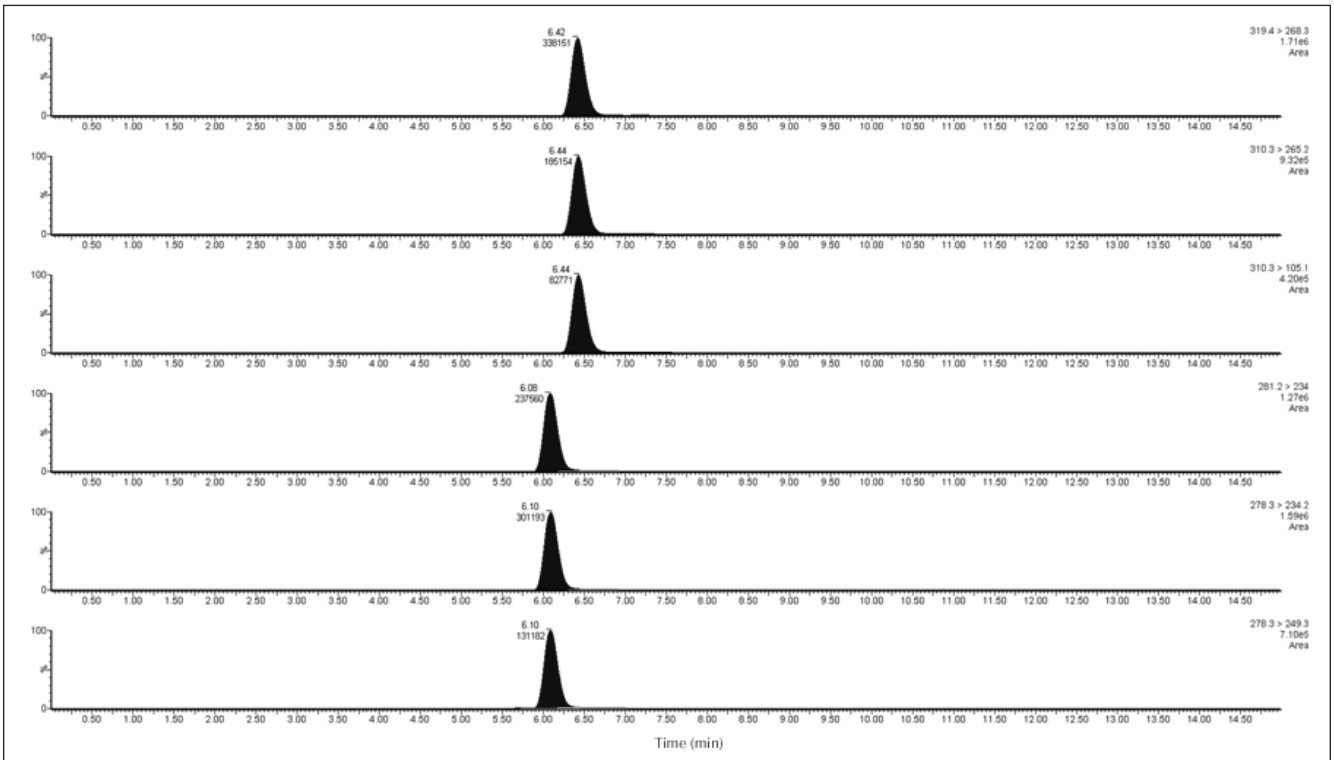

Figure 2. MRM chromatograms for (top to bottom) methadone, methadone, methadone-d_9, EDDP, EDDP, and EDDP-d_3 in a larva sampled on human remains. Detected peaks are annotated with the integrated area under the MRM chromatogram. Peak intensity and MRMs are shown on the top righthand corner of each trace.

are useful as qualitative toxicological specimens, they appear to be of limited quantitative value as the current state of research does not allow accurate quantitative assessments (4).

When analyzing insects in pupal stage instead of the larvae, only traces of methadone and EDDP were detected, and the concentration was always inferior to LOQ. For pupal stages and insect remains (exuviae or puparia) another sample preparation is necessary. In fact use of strong acids (3) or enzymatic digestion (8) for chitinized samples is recommended to break down the chitin/protein matrix, allowing the release of the drugs of interest. The dynamic range should also be altered due to the low and stable concentration of drugs present. The traces measured in pupae could be the result of bioaccumulation of metabolites at specific levels. This bioaccumulation is of interest to study the correlation between drug concentration in substrate and the post-feeding stages. This last point will be studied in further experiments.

Authentic samples

Methadone and EDDP were detected in human remains of a young man suspected to consume heroin. In a single sampled larva in feeding third instar developmental stage, we have quantified a high concentration of methadone (38.7 pg/mg larva) and EDDP (82.8 pg/mg larva) (Figure 2). This result confirms variability of drug concentration in larvae as observed in other studies such as Tracqui et al. (19). This author showed that inter-site sampling variations of drug concentrations appeared to be enormous and not reproducible. These results have also been observed by Sadler et al. (29). Although our method can detect and quantify methadone and EDDP, interpretation of the results remains difficult. Interpretation of the observed drug concentrations can be difficult due to issues such as postmortem redistribution, stability of drugs in human remains and drug interactions (19,31). In addition, a high inter- and intra-larvae sampling is important.

Conclusions

We developed and validated a method for the quantification of methadone and its metabolite EDDP in third instar larvae. This method comprises of a simple liquid–liquid extraction followed by a sensitive LC–MS–MS analysis. The method is sensitive enough to determine methadone and EDDP in one single larva. The utility of this method was confirmed through the analysis of larvae reared on a substrate that was spiked with methadone at a concentration of 4 µg/g. In addition, the method was applied to authentic postmortem material.

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